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Protein purification

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Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. Protein purification is vital for the characterisation of the function, structure and interactions of the protein of interest. The starting material is usually a biological tissue or a microbial culture. The various steps in the purification process may free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps exploit differences in protein size, physico-chemical property and binding affinity.

Contents

Purpose

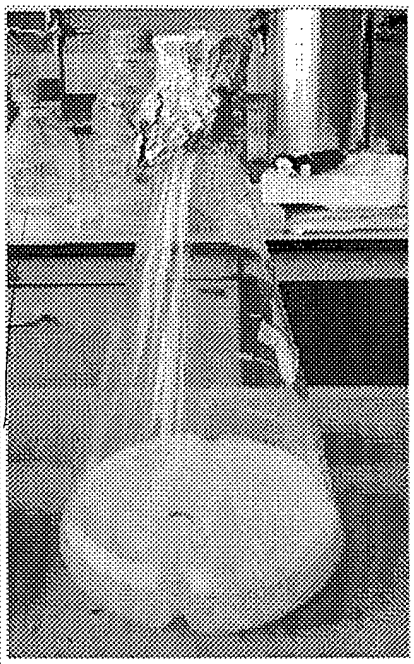
Purification may be *preparative* or *analytical*. Preparative purifications aim to produce a relatively large quantity of purified proteins for subsequent use. Examples include the preparation of commercial products such as enzymes (e.g. lactase), nutritional proteins (e.g. soy protein isolate), and certain biopharmaceuticals (e.g. insulin). Analytical purification produces a relatively small amount of protein for a variety of research or analytical purposes, including identification, quantification, and studies of the protein's structure, post-translational modifications and function. Among the first purified proteins were urease and Concanavalin A.

Strategies

Choice of a starting material is key to the design of a purification process. In a plant or animal, a particular protein usually isn't distributed

homogeneously throughout the body; different organs or tissues have higher or lower concentrations of the protein. Use of only the tissues or organs with the highest concentration decreases the volumes needed to produce a given amount of purified protein. If the protein is present in low abundance, or if it has a high value, scientists may use recombinant DNA technology to develop cells that will produce large quantities of the desired protein (this is known as an expression system). Recombinant expression allows the protein to be tagged, e.g. by a His-tag, to facilitate purification, which means that the purification can be done in fewer steps. In addition to this recombinant expression usually starts with a higher fraction of the desired protein than is present in a natural source.

An analytical purification generally utilizes three properties to separate proteins. First, proteins may be purified according to their isolectric points by running them through a pH graded gel or an ion exchange column. Second, proteins can be separated according to their size or molecular weight via size exclusion chromatography or by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis. Proteins are often purified by using 2D-PAGE and are then analysed by peptide mass fingerprinting to establish the protein identity. This is very useful for scientific purposes and the detection limits for protein are nowadays very low and nanogram amounts of protein are sufficient for their analysis.



Recombinant bacteria can be grown in a shakeflask containing growth media.

Evaluating purification yield

The most general method to monitor the purification process is by running a SDS-PAGE of the different steps. This method only gives a rough measure of the amounts of different proteins in the mixture, and it is not able to distinguish between proteins with similar molecular weight.

If the protein has a distinguishing spectroscopic feature or an enzymatic activity, this property can be used to detect and quantify the specific protein, and thus to select the fractions of the separation, that contains the protein. If antibodies against the protein is available then western blotting and ELISA can specifically detect and quantify the amount of desired protein. Some proteins function as receptors and can be detected during purification steps by a ligand binding assay, often using a radioactive ligand.

In order to evaluate the process of multistep purification, the amount of the specific protein have to be compared to the amount of total protein. The latter can be determined by the Bradford total protein assay or by absorbance of light at 280 nm, however some reagents used during the purification process may interfere with the quantification. For example, imidazole (commonly used for purification of polyhistidine-tagged recombinant proteins) is an amino acid analogue and at low concentrations will interfere with the bicinchoic acid (BCA) assay for total protein quantification. Impurities in low-grade imidazole will also absorb at 280 nm, resulting in an inaccurate reading of protein concentration from UV absorbance.

Purification of a tagged protein

Adding a tag to the protein gives the protein a binding affinity it would not otherwise have. Usually the recombinant protein is the only protein in the mixture with this affinity, which aids separation. The most common tag is the His-tag, that has affinity towards nickel ions, and thus by immobilizing nickel ions in a resin, you can make the protein bind the resin (which is packed in a column). Since the protein is the only component with a

His-tag, all other proteins will pass through the column, and leave the His-tagged protein bound to the resin. The protein is released from the column in a process called elution, which in this case involves adding imidazole, which competes with the His-tags for nickel binding. The protein of interest is now the only protein component in the eluted mixture, and can easily be separated from any minor unwanted contaminants by a second step of purification, such as size exclusion chromatography or RP-HPLC.

Other ways to tag proteins is to an antigen peptide to the protein, and then purify the protein on a column containing immobilized antibody. This generates a very specific interaction usually only binding the desired protein.

When the tags are not needed anymore, they can be cleaved off by a protease, often involving engineering a protease site between the tag and the protein.

Methods of protein purification

The methods used in protein purification, can roughly be divided into analytical and preparative methods. The distinction is not exact, but the deciding factor is the amount of protein, that can practically be purified with that method. Analytical methods aim to detect and identify a protein in a mixture, where as preparative methods aim to produce a large amount of the protein for other another purpose, such as structural biology or for industrial use. In general, the preparative methods can be used in analytical applications, but not the other way around.

Extraction

Depending on the source, the protein has to be brought into solution by breaking the tissue or cells containing it. There are several methods to achieve this: Repeated freezing and thawing, sonication, homogenization by high pressure or permeabilization by organic solvents. The method of choice depends on how fragile the protein is and how sturdy the cells are. After this extraction process soluble proteins will be in the solvent, and can be separated from cell membranes, DNA etc. by centrifugation. The extraction process also extracts proteases, which will start digesting the proteins in the solution. If the protein is sensitive to proteolysis, it is usually desirable to proceed quickly, and keep the extract cooled, to slow down proteolysis.

Precipitation and differential solubilization

In bulk protein purification, a common first step to isolate proteins is precipitation with ammonium sulfate $(NH_4)_2SO_4$. This is performed by adding increasing amounts of ammonium sulfate and collecting the different fractions of precipatate protein. One advantage of this method is that it can be performed inexpensively with very large volumes.

The first proteins to be purified were water-soluble proteins. Purification of integral membrane proteins requires disruption of the cell membrane in order to isolate any one particular protein from others that are in the same membrane compartment. Sometimes a particular membrane fraction can be isolated first, such as isolating mitochondria from cells before purifying a protein located in a mitochobrial membrane. A detergent such as sodium dodecyl sulfate (SDS) can be used to dissolve cell membranes and keep membrane proteins in solution during purification; however, because SDS causes denaturation, milder detergents such as Triton X-100 or CHAPS can be used to retain the protein's native conformation during purification.

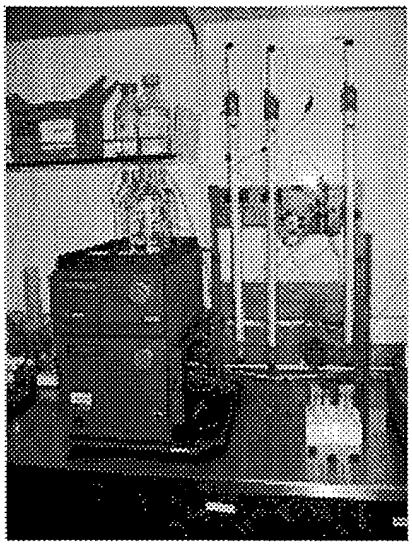
Ultracentrifugation

Centrifugation is a process that involves the use of the centrifugal force for the separation of mixtures. Increasing the effective gravitational force on a test tube so as to more rapidly and completely cause the precipitate ("pellet") to gather on the bottom of the tube. The solution ("supernatant") is then either quickly decanted from the tube without disturbing the precipitate or withdrawn by means of a medicine dropper. The rate of centrifugation is specified by the acceleration applied to the sample, typically measured in g.

Sucrose gradient centrifugation — a linear concentration gradient of sugar (typically sucrose) or Percoll is layered in a tube such that the highest concentration is on the bottom and lowest on top. The sample is then layered on top of the gradient and spun at high speeds in an ultracentrifuge. This is in turn causes heavy macromolecules to head towards the bottom of the tube faster than lighter things. The gradient in then eluted from the tube by piercing a hole in the bottom.

Chromatographic methods

Usually a protein purification protocol contains one or more chromatographic steps. The basic procedure in chromatography is to flow the solution containing the protein through a column packed with various materials. Different proteins interact differently with the column material, and can thus be separated by the time required to pass the column, or the conditions required to elute the protein from the column. Usually proteins are detected as they are coming off the column by their absorbance at 280 nm. Many different chromatographic methods exist:



Chromatographic equipment. Here set up for a size exclusion chromatography. The buffer is pumped through the column (right) by a computer controlled device.

Size exclusion chromatography

Chromatography can be used to separate protein in solution or denaturing conditions by using porous gels. This technique is known as size exclusion chromatography. The principle is that smaller molecules have to traverse a larger volume in a porous matrix. Consequentially, proteins of a certain range in size will require a variable volume of eluant (solvent) before being collected at the other end of the column of gel.

In the context of protein purification, the eluant is usually pooled in different test tubes. All test tubes containing no measurable trace of the protein to purify are discarded. The remaining solution is thus made of the protein to purify and any other similarly-sized proteins.

separation based on charge or hydrophobicity.

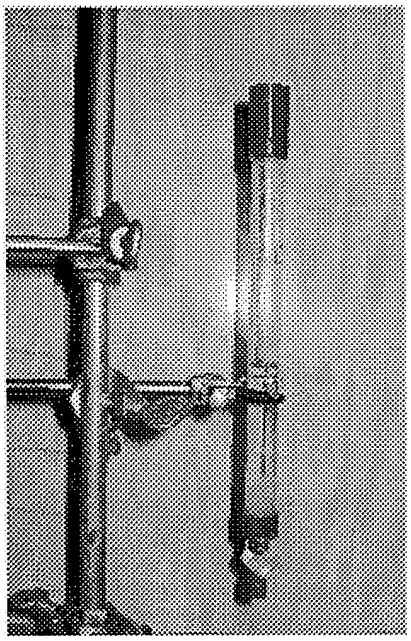
Ion exchange chromatography

Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The column to be used is selected according to its type and strength of charge. Anion exchange resins have a positive charge and are used to retain and separate negatively charged compounds, while cation exchange resins have a negative charge and are used to separate positively charged molecules.

Before the separation begins a buffer is pumped through the column to equilibrate the opposing charged ions. Upon injection of the sample, solute molecules will exchange with the buffer ions as each competes for the binding sites on the resin. The length of retention for each solute depends upon the strength of its charge. The

most weakly charged compounds will elute first, followed by those with successively stronger charges. Becauses of the nature of the separating mechanism, pH, buffer type, buffer concentration, and temperature all play important roles in controlling the separation.

Ion exchange chromatography is a very powerful tool for use in protein purification and is frequently used in both analytical and preparative separations.



A simple column for Ni^{2+}-affinity chromatography. The sample and subsequent buffers are manually poured into the column.

Affinity chromatography

Affinity Chromatography is a separation technique based upon molecular conformation, which frequently utilizes application specific resins. These resins have ligands attached to their surfaces which are specific for the compounds to be separated. Most frequently, these ligands function in a fashion similar to that of antibody-antigen interactions. This "lock and key" fit between the ligand and its target compound makes it highly specific, frequently generating a single peak, while all else in the sample is unretained.

Many membrane proteins are glycoproteins and can be purified by lectin affinity chromatography. Detergent-solubilized proteins can be allowed to bind to a chromatography resin that has been modified to have a covalently attached lectin. Proteins that do not bind to the lecting are washed away and then specifically bound glycoproteins can be eluted by adding a high concentration of a sugar that competes with the bound glycoproteins at the lectin binding site. Some lectins have high affinity binding to oligosaccharides of glycoproteins that is hard to compete with sugars, and bound glycoproteins need to be released by denaturing the lectin.

Metal binding

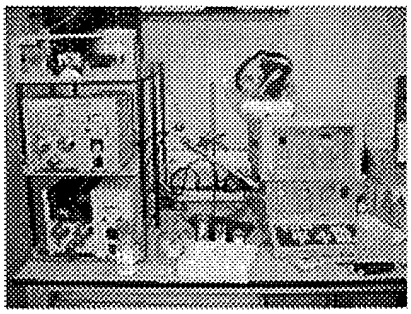
A common technique involves engineering a sequence of 6 to 8 histidines into the C-terminal of the protein. The polyhistidinebinds strongly to divalent metal ions particularly nickel. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column. The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, or by a decrease in pH (typically to 4.5), which decreases the affinity of the tag for the resin. While this procedure is generally used for the purification of recombinant proteins with an engineered affinity tag (such as a 6xHis tag or Clontech's HAT tag), it can also be used for natural proteins with an inherent affinity for divalent cations.

Immunoaffinity chromatography

Immunoaffinity chromatography uses the specific binding of an antibody to the target protein to selectively purify the protein. The procedure involves immobilizing an antibody to a column material, which then selectively binds the protein, while everything else flows through. The protein can be eluted by changing the pH or the salinity. Because this method does not involve engineering in a tag, it can be used for proteins from natural sources[1] (http://en.wikipedia.org/wiki/Protein_purification#endnote_immunoaffinity)

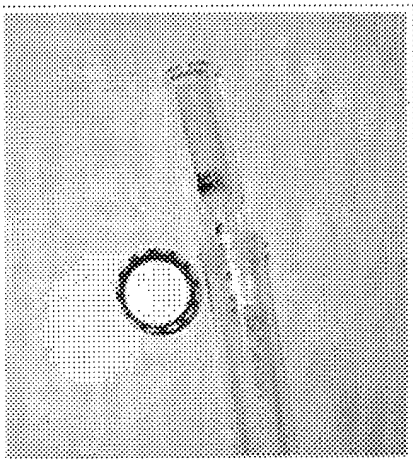
HPLC

High performance liquid chromatography or high pressure liquid chromatography is a form of chromatography applying high pressure to drive the solutes through the column faster. This means that the diffusion is limited and the resolution is improved. The most common form is "reversed phase" hplc, where the column material is hydrophobic. The proteins are eluted by a gradient of increasing amounts of an organic solvent, such as acetonitrile. The proteins elute according to their hydrophobicity. After purification by HPLC the protein is in a solution that only contains volatile compounds, and can easily be lyophilized. [2] (http://en.wikipedia.org/wiki/Protein_purification#endnote_hplc) HPLC purification frequently results in denaturation of the purified proteins and is thus not applicable to proteins that do not spontaneously refold.



A HPLC. From left to right: A pumping device generating a gradient of two different solvents, a steel enforced column and an apparatus for measuring the absorbance.

Concentration of the purified protein



A selectively permeable membrane can be mounted in a centrifuge tube. The buffer is forced through the membrane by centrifugation, leaving the protein in the upper chamber.

At the end of a protein purification, the protein often has to be concentrated. Different methods exist.

Lyophilization

If the solution doesn't contain any other soluble component than the protein in question the protein can be lyophilized (dried). This is commonly done after a HPLC run. This simply removes all volatile component leaving the proteins behind.

Ultrafiltration

Ultrafiltration concentrates a protein solution using selective permeable membranes. The function of the membrane is to let the water and small molecules pass through while retaining the protein. The solution is forced against the membrane by mechanical pump or gas pressure or centrifugation.

Analytical

Denaturing-Condition Electrophoresis

Gel electrophoresis is a common laboratory technique that can be use both as preparative and analytical method. The principle of electrophoresis relies on the movement of a charged ion in an electric field. In practice, the proteins are denatured in a solution containing a detergent (SDS). In these conditions, the proteins are unfolded and coated with negatively charged detergent molecules. The proteins in SDS-PAGE are separated on the sole basis of their size.

In analytical methods, the protein migrate as bands based on size.. Each band can be detected using stains such as Coomassie blue dye or silver stain. Preparative methods to purify large ammounts of protein, require the extraction of the protein from the electrophoretic gel. This extraction may involve excision of the gel containing a band, or eluting the band directly off the gel as it runs off the end of the gel.

In the context of a purification strategy, denaturing condition electrophoresis provides an improved resolution over size exclusion chromatography, but does not scale to large quantity of proteins in a sample as well as the late chromatography columns.

References

1. ↑ Immunoaffinity chromatography of enzymes (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=1368167&query_hl=6&itool=pubmed_DocSum) Ehle H, Horn A. Bioseparation. 1990;1(2):97-110.
2. ↑ High-performance liquid chromatography of biopolymers (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=6353575&query_hl=10&itool=pubmed_DocSum) Regnier FE Science. 1983 Oct 21;222(4621):245-52

External Links

- Protein Purification: Column Chromatography - Ion exchange; Dialysis and Concentration (http://wine1.sb.fsu.edu/bch5425/lect30/lect30.htm)
- Recombinant Protein Expression (http://www.molecularstation.com/molecular-biology-techniques/recombinant-protein-expression/)

Proteins: key methods of study

Experimental : **Protein purification** | Green fluorescent protein | Western blot | Protein immunostaining | Protein sequencing | Gel electrophoresis | Protein immunoprecipitation | Peptide mass fingerprinting

Bioinformatics : Protein structure prediction | Protein-protein docking | Protein structural alignment | Protein ontology | Protein-protein interaction prediction

Assay: Enzyme assay | Protein assay | Secretion assay

Categories: Biochemistry | Protein methods